Short Communication

Effects of phenidone (DuCLOX-2/5 inhibitor) against N-methyl-N-nitrosourea induced mammary gland carcinoma in albino rats

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\textbf{ABSTRACT}

The present study was designed to evaluate the effects of phenidone (Dual inhibitor of COX-2 and 5-LOX, DuCLOX-2/5 inhibitor) on various aspects of cancer chemoprevention. Treatment with the phenidone was inquired to validate the implications of dual inhibition of arachidonic acid (AA) metabolism against MNU induced mammary gland carcinogenesis. MNU treated rats showed altered hemodynamic profile, distorted cellular architecture, upregulated inflammatory enzyme markers (COX, LOX, Nitric oxide and hydrogen sulfide) and distorted oxidative stress markers (thioibarbituric acid reactive substances, protein carbonyl, superoxide dismutase, catalase and glutathione). Phenidone treatment regulated histological architecture in the experimental animals similar to control. The treatment with phenidone favorably regulated the levels of inflammatory markers, and oxidative stress markers against toxic treatment. Our findings emphasize the potential role of phenidone in suppression of mammary gland carcinoma against the deleterious effects of MNU.

1. Introduction

Emerging reports have demonstrated an association between inflammation and cancer development or augmentation. Arachidonic acid (AA) and its metabolites eicosanoids are associated with a more advanced disease stage at the time of diagnosis of human cancer (Yang et al., 2011; Greene et al., 2011). AA is an essential fatty acid and a member of the omega-6 (n-6) polyunsaturated fatty acids (PUFA) that plays imperative role in inflammation, and cellular metabolism and signaling. Deregulation of the eicosanoids plays essential role in inflammation, and its associated diseases including human cancer (Gautam et al., 2016; Tavolari et al., 2007; Ye et al., 2005).

AA released from cell membrane is metabolized from two vital enzymes, namely cyclooxygenase (COX) (two isoforms, COX-1 and COX-2) and lipoxygenase (LOX) (including 5-LOX, 12-LOX and 15-LOX). Induction of COX-2 by inflammatory stimulus results in formation of PGE\textsubscript{2}, PGF\textsubscript{2α}, PGB\textsubscript{2}, PGI\textsubscript{2}, TXA\textsubscript{2} and TXB\textsubscript{2}, and their involvement in carcinogenesis is well documented. COX-2 and its metabolic products, for instance, PGE\textsubscript{2} has been found to be overexpressed in the several human cancers including breast, colon, lungs and pancreas. A large body of study indicates that overexpression of COX-2 and PGE\textsubscript{2} during carcinogenesis is implicated in proliferation, invasion, apoptosis, immune suppression, and angiogenesis (Gautam et al., 2016; Wang and DuBois, 2008). Additionally, published reports have also endorsed that LOX inflammatory pathway hinders the normal physiological function of the cells and in particular, 5-LOX metabolic products act as a mediator of alteration of cell. Cancer cell proliferation, apoptosis, angiogenesis and invasiveness mediated by the leukotrienes suggests that 5-LOX pathway has a stimulus on the development of cancers (Ghosh and Myers, 1997; Kim et al., 2005).

Epidemiological and preclinical studies indicate that the intermittent co-expression of these two enzyme and their indigenous biological functions can drive tumour progression. More recent evidence suggests that the drugs which are able to block the inflammatory cascade, in particular dual inhibitors of COX-2 and 5-LOX, may help in the regulation of the carcinogenesis and tumour development (Tavolari et al., 2007; Altavilla et al., 2012). Based on the above evidences, a dual inhibitor of COX-2 and 5-LOX, phenidone, was implicated in the present

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study inquesting for the regulation of the breast carcinoma and for the development of new therapeutic approach for breast cancer therapy. Phenidone, which belongs to Pyrazolidinone class primarily demonstrated variety of biological activities such as anti-inflamatory, hypotensive effect and have effect on acute mild to moderate pain.

Hereupon, on a similar line of DuCLOX-2/5 inhibition, the present study was proposed to scrutinized the potential chemopreventive effects of phenidone on preclinical model of methyl nitrosourea (MNU) induced mammary gland carcinoma.

2. Materials and methods

2.1. Drugs and chemicals

Phenidone was solicited as API from M.P. Chemicals Ltd., India, and Tamoxifen was procured from local market under the brand name Tamodex from Biochem pharmaceutical industries Ltd, India. N-Nitroso-N-Methylurea (MNU) was retrieved from Sigma Life Science Aldrich USA. All other chemicals were of analytical grade and procured from Hi-media Laboratories Mumbai, India; else otherwise stated in the text.

2.2. Animals

Albino wistar female rats of 100–120 g body weight were used for this study. The rats were procured from the central animal house facility. The animals housed in polyplypropylene cages under controlled conditions (23 °C, 12 h light/dark cycle). Animal experiments were carried out in compliance with the standard ethical guidelines and approved by Institutional Animal Ethics Committee (IAEC/SHIATS/17MBM12).

2.3. Experimental protocol

Animals were randomized and divided into 5 groups of 6 animals each. Group I - normal control (1% cmc, p.o.); Group II- toxic control (MNU 47 mg/kg, i.v.); Group III - Standard control (Tamoxifen 1 mg/kg, p.o. + MNU 47 mg/kg, i.v.); Group IV - Phenidone Low dose treatment (Phenidone, 5 mg/kg, p.o. + MNU 47 mg/kg, i.v.); Group V - Phenidone High dose treatment (Phenidone, 10 mg/kg, p.o. + MNU 47 mg/kg, i.v.). Mammary gland carcinogenesis was induced by single i.v. injection of MNU (Gautam et al., 2018) followed by anti-inflammatory doses of phenidone (Calixto et al., 1991) for 90 days. Anticancer drug tamoxifen was preferentially used as a standard control, as it has been approved for its established chemopreventive efficacy and known to reduce risk of estrogen receptor positive (ER +) breast cancer (Wakeling and Valcacia, 1983). The blood samples were collected from animals under chloroform anaesthesia through retro orbital plexus. The blood samples were incubated at 37 °C for 1 h and centrifuged at 10,000 rpm for 15 min to collect serum. The serum samples were stored at −20 °C till further use. Animals were sacrificed on the 90th day and mammary gland tissue was collected for estimations.

2.4. Hemodynamic changes

The animals were anesthetized using ketamine hydrochloride (100 mg/kg, i.m.) and diazepam (5 mg/kg, i.m.) in combination and subsequently mounted on a wax tray (on 89th day). The platinum hook electrodes were placed on the skin of the dorsal and ventral thorax to record the electrocardiogram (ECG) signal. The electrodes were connected to Bio-amplifier (ML-136) and channel PowerLab (ML-826) to convert analogue to digital signals (AD Instruments, Australia). The ECG signals were used to perform heart rate variability (HRV) analysis and analyzed using Labchart Pro-8 (AD Instruments, Australia) (Caro-Morán et al., 2016).

2.5. Carmine staining of whole mounts mammary gland

Mammary gland tissues from each group were assessed for their morphological changes using method previously established at our laboratory (Manral et al., 2016; Rani et al., 2016). Whole mounts of the mammary gland tissues were examined under the microscope and evaluated to assess the number terminal end buds (TEBs), alveolar buds (AB) (type 1 and 2), and differentiation (DF) score (Rani et al., 2016).

2.6. Morphological evaluation

Mammary gland tissues from each group were appraised histopathologically using haematoxyline and eosin staining using a well-established method at our laboratory (Roy et al., 2017; Murray et al., 2009).

2.7. Biochemical estimation

The mammary gland tissues (10% w/v) were homogenized in 0.15 M KCl and centrifuged at 10,000 rpm. The supernatants were scrutinized for biochemical parameters including thiobarbituric acid reactive substances (TBARs), superoxide dismutase (SOD), catalase, Protein carbonyl (PC), glutathione (GSH), using the methods established at our laboratory (Kaithwas et al., 2007; Reznick and Packer, 1994).

2.8. Serum nitric oxide (NO) level

Generation of NO in the serum samples were estimated by measuring nitrite accumulation, using Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% H2PO4). Equal quantity of serum and griess reagent were mixed and incubated at 37 °C for 5 min. The test mixture was subsequently read on UV-Visible spectrophotometer (Cary60, Agilent technologies, CA95051, US) at 540 nm (Rossi and Tiskas, 2009).

2.9. Plasma H2S

Methylene blue method as prescribed by Shen et al. was used for the determination of serum H2S level with slight modification. 75 μl of serum sample was added to 425 μl of distilled water and 250 μl of 1% (w/v) zinc acetate. 135 μl of 20 mM N,N-dimethyl-p-phenylenediamine (1-napthyl)-ethylenediamine dihydrochloride in 5% H2PO4. Equal quantity of serum and Griess reagent were mixed and incubated at 37 °C for 10 min. The test mixture was subsequently read on UV-Visible spectrophotometer (Cary60, Agilent technologies, CA95051, US) at 540 nm (Rossi and Tiskas, 2009).

2.10. Enzymatic activity of COX and LOX

The serum was incubated for 5 min with tris buffer (160 μl). A 10 μl each of 200 μM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) reagent and 20 μM arachidonic acid (AA) solution were added and read at 630 nm using multiplate reader (ALERE Microplate Reader, AM-2100) at 0 and 30 s interval (Cullen et al., 1998).

For LOX assay, 25 μl of 20 μM AA solution was added to the 475 μl serum supernatant and incubated for 6 min. A 500 μl of ferrithiocyanate (FTC) reagent was added and read at 480 nm using UV visible spectrophotometer (Cary 60, Agilent Technologies International Private Limited, CA United States) after 5 min. FTC reagent was prepared by mixing the equal volume of reagent 1 (4.5 mM FeSO4 in 0.2 M HCl) and reagent 2 (3% NH4SCN methanolic solution) (Lu et al., 2013).
Fig. 1. Representative ECG recordings of the control, MNU and phenidone treated animals. Recording of the ECG of the individual groups, Control (1% cmc, p.o.) (I), Toxic control (MNU, 47 mg/kg, i.v.) (II), Tamoxifen (Tamoxifen 1 mg/kg, p.o. + MNU 47 mg/kg, i.v.) (III), Phenidone (5 mg/kg, p.o. + MNU 47 mg/kg, i.v.) (IV) and phenidone (10 mg/kg, p.o. + MNU 47 mg/kg, i.v.) (V).
2.11. Statistical analysis

All data were presented as mean ± SD and analyzed by one way ANOVA followed by Bonferroni’s multiple comparison test for the possible significance identification between the various groups. α/4 < 0.05, β/4 < 0.001, c/4 < 0.001 were considered statistically significant. Statistical analysis was carried out using Graph Pad Prism (5.01), San Diego, California.

3. Results

The oral administration of phenidone in MNU treated albino rats significantly affected the ECG parameters (Fig. 1). MNU administration aberrantly distorted ECG profiling characterized by QT interval (0.07 ± 0.07), QRS prolongation (0.02 ± 0.01), sizeable amount of decrease in HR (376.76 ± 12.07) and increased dispersion of P wave (0.015 ± 0.01) in comparison to control. The same was restored towards normal after phenidone administration (Supplemental Table S1).

Distorted HRV profile was recorded for the time domain (Average RR, Median RR, SDRR, SDARR and CVRR) and frequency domain (LF, HF and LF/HF) after MNU treatment. Treatment with phenidone exerted favorable effects towards restoring the HRV paradigms towards normal (Supplemental Table S2).

The results revealed abrupt inflation in the AB (486.75 ± 17.17) and lobule score (551.0 ± 34.22) with MNU treatment which was dose dependently curtailed down with the phenidone treatment. Similar pattern of significant results were evident in the phenidone while scrutinizing the DF score in the whole mounts of the mammary gland (Table 1) (Fig. 2). The MNU treatment distorted the normal architecture of the mammary gland with loss of LCT and DCT along with myoe-dipithelial cells, goblet epithelial cells while examining through H&E. Phenidone treatment restored the normal architecture in the dose dependent manner (Fig. 2).

Administration of MNU made an evident upsurge in TBARS level in comparison to normal control. The phenotype at both doses significantly curtailed down TBARS level along with standard group. Upreregulated SOD (0.04 ± 0.001) and catalase (0.90 ± 0.03) was observed on MNU treatment in comparison to control which was restored through phenidone treatment. Similar pattern of results were scrutinized for the GSH. Upsurged PC levels were observed in the MNU treated animals and the same was restored on treatment with phenidone (Table 2).

Treatment with MNU upregulated the COX and LOX enzymatic activity in mammary gland tissue and phenidone treatment afforded significant curtailment of the same in dose dependent manner. Similar patterns of negating effects were observed while scrutinizing the NO and H2S levels in treatment group against MNU treatment (Table 2).

4. Discussion

The present study demonstrated the effect of phenidone against MNU induced mammary gland carcinogenesis in albino wistar rats. The whole mount preparations of mammary gland tissue are frequently used as a convenient method for the examination of small proliferative lesions as represented through lobules and increase in number of AB1/TEB count. These undifferentiated AB1/TEB and lobules are the sites for the malignant transformations (Gautam et al., 2018; Lamartiniere et al., 1995). The MNU treatment was evident with increase in AB count, DF score and lobules number. Treatment with phenidone curtailed down the AB count and DF score to sizable amount in dose dependent manner, suggesting positive modulatory effect of phenidone against MNU induced mammary gland differentiation, as in line with the previous literature (Table 1). To validate our findings, mammary gland tissues were further scrutinized histopathologically. Mammary gland tissues were examined for presence of duct, adipocytes, loose connective tissue (LCT), dense connective tissue (DCT), epithelial cells and lymphocytes. The results revealed significant inflation in the distorted cellular morphology, with scattered cuboidal epithelial cells and loss of adipocytes after MNU treatment in toxic control group. Treatment with phenidone significantly restored the morphological architecture compared with toxic control group (Liska et al., 2000) (Fig. 2).

In the present study, the treatment with both doses of phenidone revert back heart rate to normal. HRV and ECG is a common indicator of Autonomic nervous system (ANS) function and delivers a multidimensional element of ANS through sympathetic and parasympathetic variation of cardiac activity (Caro-Morán et al., 2016; Meinardi et al., 2001). In our present study, the treatment with high dose of phenidone restored the HRV in the time domain and frequency domain in comparison to MNU treated animals (Supplemental Tables S1 & S2).

Release of NO by macrophages and epithelial cells on inflammation results in nitrosylation of cellular proteins and DNA damage. Their excessive overexpression has been linked to several neurodegenerative, immune and inflammatory disorders, and has been well reported in various inflammatory induced carcinogenesis. Moreover, we observed significant increase in the enzymatic activity of COX and LOX enzymes, which was further accomplished by inhibited NO production in the mammary gland tissues. Treatment with both doses of phenidone helped to restore the enzymatic activity of COX and LOX towards normal. In the current experiment, increased enzymatic activity of COX-2 and 5-LOX after the MNU treatment affirmed the association between inflammation and carcinogenesis. Treatment with phenidone regulated the overexpression of NO levels in a prevailing study and is hypothesised that increased NO generation in a cell may contribute to tumour angiogenesis by upregulating vascular endothelial growth factor (VEGF) (Bustamante et al., 2002; Muntané and De la Mata, 2010). On a similar scale, inflated NO levels subsequent to MNU treatment can be foreseen as a marker of cancer progression and is in line with various preclinical studies. To put additional affirmation,

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>AB1 (1% cmc, p.o.)</th>
<th>AB2</th>
<th>AB (AB1 + AB2)</th>
<th>Lobules</th>
<th>DF. score (AB1 + AB2 + lobules)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1% cmc, p.o.)</td>
<td>189.75 ± 12.68***</td>
<td>107.25 ± 14.81***</td>
<td>297 ± 21.02***</td>
<td>319.5 ± 21.11***</td>
<td>616.5 ± 41.74***</td>
</tr>
<tr>
<td>Toxic control (MNU, 47 mg/kg, i.v.)</td>
<td>312.75 ± 14.26</td>
<td>174.0 ± 12.41</td>
<td>486.75 ± 17.17</td>
<td>551.0 ± 34.22</td>
<td>1037.75 ± 42.08</td>
</tr>
<tr>
<td>Tamoxifen (1 mg/kg, p.o.) + (MNU, 47 mg/kg, i.v.)</td>
<td>213.75 ± 24.99***</td>
<td>122.75 ± 12.32***</td>
<td>363.5 ± 15.46***</td>
<td>386.0 ± 27.38***</td>
<td>722.5 ± 48.21***</td>
</tr>
<tr>
<td>Phenidone (5 mg/kg, p.o.) + (MNU, 47 mg/kg, i.v.)</td>
<td>208.0 ± 14.16***</td>
<td>140.25 ± 6.65***</td>
<td>348.25 ± 15.73***</td>
<td>384.75 ± 40.96***</td>
<td>733.0 ± 40.65***</td>
</tr>
<tr>
<td>Phenidone (10 mg/kg, p.o.) + (MNU, 47 mg/kg, i.v.)</td>
<td>193.0 ± 28.34***</td>
<td>109.25 ± 9.03***</td>
<td>302.25 ± 35.02***</td>
<td>318.75 ± 19.91***</td>
<td>621.0 ± 24.58***</td>
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(Values are presented as Mean ± SD, each group contains 6 animals. Comparisons were made on the basis of the one-way ANOVA followed by Bonferroni multiple test. All groups were compared to the toxic control group (*p < 0.05, **p < 0.01, ***p < 0.001). Tamoxifen group was compared to the phenidone treated groups (*p < 0.05, *p < 0.01, **p < 0.001).
plasma $\text{H}_2\text{S}$ signaling was studied in experimental animals. Elevated Plasma $\text{H}_2\text{S}$ levels in toxic MNU are in corroboration with the previous reports (Szabo et al., 2013) and in line with the above findings. We perceived significant curtailment in the level of $\text{H}_2\text{S}$ after phenidone treatment, thereby reflecting protective effect of phenidone on cancer cells (Table 2).

Cancer progression is ascertained to be concorded with sizable deterioration in the oxidative stress markers and the same was evident in the present study. The peroxidation products of the oxidative damaged pathways are TBARs and PC (by product of amino acids and protein) (Rani et al., 2016; Verma et al., 2016). TBARs are stable lipid peroxidation biomarker and PC is a protein peroxidation biomarker respectively. In the present study, we observed a momentous increase in both the biomarkers in toxic groups. Both the doses of treatment were found to be effective which lowered the TBARs level and PC back to normal depicting efficacy. In addition, antioxidant enzymes Catalase and SOD work in conjugation catalytically remove free radicals and ROS (Kaithwas et al., 2007; Trachootham et al., 2009). We observed the decrease in SOD and catalase levels in animals subjected to treatment with both doses of phenidone. Investigation also concluded that treatment with test drug found to elevate the levels of tissue GSH as compared to toxic control. GSH is a marker of short term oxidative

**Fig. 2.** Microscopic evaluation of the mammary gland tissue of the animal treated with phenidone using Carmine staining and H&E staining.
Whole mount carmine staining of ductal epithelium reveals the presence of Alveolar bud (1) and lobules (2) in respective groups (A, B, C D and E). The extent of alveolar budding and lobule formation are excessive in toxic group (B) which has been subsided with respective treatment groups (D and E). The images were captured under microscope with $4\times$ magnification. Histopathological evaluation of the mammary gland tissue with H&E staining revealed the presence of duct (3), adipocytes (4), LCT (5), DCT (6), MEC (7), lymphocytes (8) and CEC (9) in control (F), as well as tamoxifen (H) and phenidone (low and high dose) (I&J) treated groups respectively. Absence of cell organelles and distorted cell morphology was found in toxic group (G). The images were captured under microscope with $40\times$ magnification.
Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein carbonyl (nM/ml unit)</th>
<th>Catalase (units of H2O2 disapp. min/mg of protein)</th>
<th>SOD (units of SOD/μg of protein)</th>
<th>TBARs (nM of MDA/μg of protein)</th>
<th>NO (μM/ml unit)</th>
<th>COX (percentage inhibition)</th>
<th>LOX (percentage inhibition)</th>
<th>GSH (μg %)</th>
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<tr>
<td>Control (1% cmc p.o.)</td>
<td>479.70 ± 56.44***</td>
<td>0.66 ± 0.01</td>
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<td>Toxic control (MNU, 47 mg/kg, i.v.)</td>
<td>698.56 ± 89.97**</td>
<td>0.60 ± 0.01</td>
<td>0.03 ± 0.01</td>
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<td>Tamoxifen (1 mg/kg, p.o.) + MNU (47 mg/kg, i.v)</td>
<td>199.15 ± 83.23***</td>
<td>0.66 ± 0.01</td>
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<td>Phenidone (5 mg/kg, p.o.) + MNU (47 mg/kg, i.v)</td>
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References


Greene, E.R., Huang, S., Serhan, C.N., Panigrahy, D., 2011. Regulation of inflammatory stress and was found low in toxic group due to this utilization to combat free radical formation by MNU.

With all above, one can conclude that phenidone can impart significant protection against MNU induced mammary gland carcinoma in dose dependent manner. The study can be advocated for pivot role of DuCLOX-2/5 inhibition for chemopreventive target for mammary gland carcinogenesis. Further studies will be needed to confirm the appropriateness of the use of DuCLOX-2/5 inhibition in particular for prevention of human breast cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2018.04.019.

Conflict of interest

Authors declare no competing interest.

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